Inhibition of Microtubule Assembly in Tumor Cells by 3-Bromoacetylaminobenzoylurea, a New Cancericidal Compound

Jian-Dong Jiang, Yue Wang, John Roboz, James Strauchen, James F. Holland, and J. George Bekesi

Division of Neoplastic Diseases, Department of Medicine, Mount Sinai School of Medicine, New York, New York 10029

ABSTRACT

We have synthesized a new compound, 3-bromoacetylaminobenzoylurea (3-BAABU), which showed strong cancericidal activity by inducing irreversible mitotic arrest and subsequently apoptosis in human T cell leukemia cells (CEM), human biphenotypic leukemia cells (SP), a human prostate cancer cell line (PC-3), murine melanoma cells (B16), and murine lymphoma/leukemia cells (EL4) in vitro with an ID50 in the range of 0.013-0.07 μg/ml (0.04-0.22 μM). Treatment of tumor cells for 12-24 h with 3-BAABU resulted in mitotic arrest at prometaphase/metaphase/ anaphase, with separation and dispersion of chromosomes and with the absence of mitotic spindle apparatus in cytoplasm. Treatment with 3-BAABU had no cytotoxic and mitotic blocking effect in normal human myocardial cells, proliferating fibroblast cells (3T3), or proliferating myocardial cells (MOT). Cell cycle analyses showed that most treated leukemic cells accumulated at M phase 12 h after treatment. By the end of 48 h of treatment, the cells underwent apoptosis with DNA fragmentation. 3-BAABU inhibited the assembly of microtubules from tubulin but did not interfere with the disassembly of microtubules. The presence and the position of bromine and urea groups on the benzoic ring are the determining factors for its inhibition of microtubule assembly. Replacing bromine with chlorine yielded much less mitotic blocking activity and increased the ID50 40-fold. Substitution of the urea group with ethyl ester abrogated the activity of blocking mitosis but induced apoptosis. Moving the bromoacetylaminogroup from the 3-position to the 4-position resulted in sensitized microtubule disassembly activity for mitosis but induced necrosis. These results suggest that 3-BAABU possesses a unique and functional structure and is a potential agent for cancer chemotherapy.

INTRODUCTION

Microtubules play important roles in a variety of biological functions, especially in governing the movement of chromosomes during mitosis. Components of this biological process are targets for a group of compounds including vinblastine (1), vincristine (1), colchicine (2), podophyllotoxin (3), paclitaxel (4), and docetaxel (5), which exert mitotic blocking activity by interfering with the exchange of tubulin subunits between the microtubules and the free tubulin pool in cytoplasm (6). These agents are large dimeric natural complexes from plants or mimic synthetic variants. Increased knowledge of the molecular targets for regulation of cell growth as well as of the functions of checkpoints in the cell cycle (6-8) prompted us to design and synthesize new compounds of simple structure and low molecular weight to regulate cell cycle. The representative compound of this group is 3-BAABU, which interferes with the repolymerization of microtubules from free tubulin in tumor cells, blocks the cell cycle in mitosis, and eventually leads to apoptotic cell death. Induction of mitotic arrest at the doses tested is restricted to malignant cells; normal lymphocytes, proliferating fibroblasts, and rapidly growing myocardial cells were unaffected. This communication reports our observations in vitro using this compound.

MATERIALS AND METHODS

Reagents. 3-BAABU and its analogues 3-CAABU, 3-BAABE, 4-BAABU, and 3-BPABU were synthesized in our laboratory (patent pending). Structures of these compounds (see Table 1) were confirmed by mass spectrometry and nuclear magnetic resonance. Details of the synthesis and confirmation of the structures of these compounds (Table 1) and some other haloacetyl analogues will be described elsewhere. All compounds were dissolved in a mixture of N,N-dimethyl acetamide, propylene glycol, and Tween 80 (1:2:1, v/v/v) and were further diluted in culture medium prior to use. Paclitaxel and vinblastine sulfate (Aldrich Chemical Co., Milwaukee, WI) were dissolved in DMSO before use. Equal volumes of solvents were used in the control experiments.

Cells. All cell lines, with the exception of human SP cells, a biphenotypic leukemic cell line (9, 10), were obtained from the American Type Culture Collection (Rockville, MD) and were cultured under conditions recommended by the vendor. EL4 murine leukemia/lymphoma (as a suspension) and B16 mouse melanoma (as a monolayer) cell lines were cultivated in RPMI 1640 supplemented with 10% FBS, penicillin (250 units/ml), and streptomycin (250 units/ml); MOT, a nonmalignant and proliferative myocardial cell line from rat, and NIH 3T3, a nonmalignant and proliferative fibroblast cell line from mouse, were both cultured as monolayers in DMEM with 10% heat-inactivated calf serum. CEM, a human acute T-cell leukemic cell line, was cultured as a suspension in Iscove’s Dulbecco’s medium with 10% heat-inactivated FCS and antibiotics. PC-3, a human prostate cancer line, was cultured (as monolayers) in RPMI 1640 with 10% FBS. SP cells were cultured in minimum Eagle’s medium plus 10% FBS. Human PBLs were isolated from whole blood of healthy individuals by Ficoll-Hypaque gradient. Isolated PBL cells were maintained in RPMI 1640 in the presence of 10% homologous plasma at 37°C. All cells were incubated in 5% CO2 at 37°C. Cells in exponential growth were used.

Cytotoxicity, ID50, and ID90 Determination. Cells in suspension culture were seeded into 96-well microplates (Falcon, Oxnard, CA) at 103 cells/well, followed by treatment with 0-10 μg/ml of the candidate compounds at 37°C for 48 h. Total volume was 250 μl/well. Cell viability was assessed by trypan blue exclusion.

For the monolayer cultures, cells were placed in 96-well microplates at 5 × 104 cells/well (250 μl/well) in the presence or absence of candidate compounds at concentrations between 0 and 10 μg/ml and incubated at 37°C for 48 h. To obtain homogeneous single-cell suspensions for staining and counting, the supernatants were removed gently, and the cells left in the wells were digested with EDTA-Trypsin. ID50 and ID90 were determined in duplicate in every set of experiments, and each experiment was repeated three times under identical conditions. ID50 and ID90 were defined as drug concentrations that induced 50 or 90% cellular death in comparison with untreated controls and calculated by nonlinear regression analysis.

Cancericidal Activity after Pulse Exposure. CEM cells were pulse exposed to 3-BAABU, 0.025 μg/ml at 37°C for 15, 30, 60, 120, and 240 min, respectively. After incubation, cells were washed twice in PBS. Cell pellets were resuspended in drug-free medium and incubated at 37°C for 48 h. Cell viability was determined 48 h post-pulse exposure.

cells were identified using previously defined criteria, i.e., shrinkage in cell dry pellet of nucleic acid was resuspended in a solution of 10 mM Tris-HCl/1 (24:1, v/v), and precipitated with ethanol followed by centrifugation at 7800 g. The resulting products were extracted with phenol and chloroform:isoamylalcohol (1:24:1, v/v) containing 1 µg/ml protease K for 1 h at 50°C. The destruction of the nuclei.

Morphology. Cell samples on slides were prepared by a Cytospin centrifuge (Shandon Southern Products Ltd, England) at 700 x g for 5 min. The slides were air dried, fixed in methanol, and stained with Giemsa (Harleco, Gibbstown, NJ) at room temperature for 15 min. Alternations of nuclei, membranes, and morphological features were observed by light microscopy. Cells in mitotic phase were recognized by the appearance of chromosomes dispersed in the cytoplasm and by the disappearance of nuclear membranes. Apoptotic cells were identified using previously defined criteria, i.e., shrinkage in cell dry pellet of nucleic acid was resuspended in a solution of 10 mM Tris-HCl/1 (24:1, v/v), and precipitated with ethanol followed by centrifugation at 7800 g.

DNA Fragmentation. Soluble DNA from cells was extracted by a previously reported method (13). Briefly, after washing with PBS, cells were treated in 1 ml of lysis buffer (0.01 mM Tris-HCl, pH 8.0, 0.01 mM NaCl, 0.01 mM EDTA, pH 8.0, and 5% SDS) containing 1 µg/ml protease K for 1 h at 50°C. The resulting products were extracted with phenol and chloroform:isoamylalcohol (24:1, v/v), and precipitated with ethanol followed by centrifugation at 7800 g.

Table 1: The structure-activity relationship of 3-BAABU in CEM leukemic cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular weight</th>
<th>Cytotoxicity (ID50) µg/ml</th>
<th>Inhibition of MT assembly (ID50) µg/ml</th>
<th>Inducing activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-BAABU</td>
<td>253</td>
<td>0.15</td>
<td>0.59</td>
<td>NE</td>
</tr>
<tr>
<td>3-BAABU</td>
<td>300</td>
<td>0.013</td>
<td>0.04</td>
<td>+ + +</td>
</tr>
<tr>
<td>3-BAABU</td>
<td>300</td>
<td>0.07</td>
<td>0.23</td>
<td>NE</td>
</tr>
<tr>
<td>3-BAABU</td>
<td>256</td>
<td>0.50</td>
<td>1.95</td>
<td>&gt;25</td>
</tr>
<tr>
<td>3-BAABU</td>
<td>314</td>
<td>NT</td>
<td>NT</td>
<td>NE</td>
</tr>
</tbody>
</table>

For activity assays, see “Materials and Methods.” Mitotic block was evaluated at 24 h, and apoptosis and necrosis were determined at 48 h. The level of activities are expressed by % of affected cells. NE, not effective up to 50 µg/ml; MT, microtubule; NT, not toxic up to 10 µg/ml.

Morphology. Cell samples on slides were prepared by a Cytospin centrifuge (Shandon Southern Products Ltd, England) at 700 x g for 5 min. The slides were air dried, fixed in methanol, and stained with Giemsa (Harleco, Gibbstown, NJ) at room temperature for 15 min. Alternations of nuclei, membranes, and morphological features were observed by light microscopy. Cells in mitotic phase were recognized by the appearance of chromosomes dispersed in the cytoplasm and by the disappearance of nuclear membranes. Apoptotic cells were identified using previously defined criteria, i.e., shrinkage in cell dry pellet of nucleic acid was resuspended in a solution of 10 mM Tris-HCl/1 (24:1, v/v), and precipitated with ethanol followed by centrifugation at 7800 g.

DNA Fragmentation. Soluble DNA from cells was extracted by a previously reported method (13). Briefly, after washing with PBS, cells were treated in 1 ml of lysis buffer (0.01 mM Tris-HCl, pH 8.0, 0.01 mM NaCl, 0.01 mM EDTA, pH 8.0, and 5% SDS) containing 1 µg/ml protease K for 1 h at 50°C. The resulting products were extracted with phenol and chloroform:isoamylalcohol (24:1, v/v), and precipitated with ethanol followed by centrifugation at 7800 g.

Table 1: The structure-activity relationship of 3-BAABU in CEM leukemic cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular weight</th>
<th>Cytotoxicity (ID50) µg/ml</th>
<th>Inhibition of MT assembly (ID50) µg/ml</th>
<th>Inducing activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-BAABU</td>
<td>253</td>
<td>0.15</td>
<td>0.59</td>
<td>NE</td>
</tr>
<tr>
<td>3-BAABU</td>
<td>300</td>
<td>0.013</td>
<td>0.04</td>
<td>+ + +</td>
</tr>
<tr>
<td>3-BAABU</td>
<td>300</td>
<td>0.07</td>
<td>0.23</td>
<td>NE</td>
</tr>
<tr>
<td>3-BAABU</td>
<td>256</td>
<td>0.50</td>
<td>1.95</td>
<td>&gt;25</td>
</tr>
<tr>
<td>3-BAABU</td>
<td>314</td>
<td>NT</td>
<td>NT</td>
<td>NE</td>
</tr>
</tbody>
</table>

For activity assays, see “Materials and Methods.” Mitotic block was evaluated at 24 h, and apoptosis and necrosis were determined at 48 h. The level of activities are expressed by % of affected cells. NE, not effective up to 50 µg/ml; MT, microtubule; NT, not toxic up to 10 µg/ml.

Morphology. Cell samples on slides were prepared by a Cytospin centrifuge (Shandon Southern Products Ltd, England) at 700 x g for 5 min. The slides were air dried, fixed in methanol, and stained with Giemsa (Harleco, Gibbstown, NJ) at room temperature for 15 min. Alternations of nuclei, membranes, and morphological features were observed by light microscopy. Cells in mitotic phase were recognized by the appearance of chromosomes dispersed in the cytoplasm and by the disappearance of nuclear membranes. Apoptotic cells were identified using previously defined criteria, i.e., shrinkage in cell dry pellet of nucleic acid was resuspended in a solution of 10 mM Tris-HCl/1 (24:1, v/v), and precipitated with ethanol followed by centrifugation at 7800 g.

DNA Fragmentation. Soluble DNA from cells was extracted by a previously reported method (13). Briefly, after washing with PBS, cells were treated in 1 ml of lysis buffer (0.01 mM Tris-HCl, pH 8.0, 0.01 mM NaCl, 0.01 mM EDTA, pH 8.0, and 5% SDS) containing 1 µg/ml protease K for 1 h at 50°C. The resulting products were extracted with phenol and chloroform:isoamylalcohol (24:1, v/v), and precipitated with ethanol followed by centrifugation at 7800 g.
Solvent solutions, used as a control, had no effect in either the assembly or disassembly process.

RESULTS

Blocking of Mitosis in Tumor Cells by 3-BAABU. Table 1 shows the structures of the compounds studied, ID₅₀ values and the degree of their activities in inducing mitotic block, apoptosis, and necrosis in leukemic cells. 3-BAABU showed powerful antileukemia activity, with an ID₅₀ of 0.013 μg/ml. When bromine was substituted with chlorine forming 3-CAABU, the ID₅₀ increased 40-fold with respect to 3-BAABU. Replacing the urea group with ethyl ester (3-BAABE) increased the ID₅₀ 10-fold. Moving the bromoacetylamino group from the meta- to the para-position (4-BAABU) kept the ID₅₀ similar to that of 3-BAABU. Extending the acetylamino chain with one more methylene group, forming 3-BPABU, eliminated cytotoxic activity.

The morphology of CEM cells treated (for 24 h) with quantities corresponding to the respective ID₅₀ of 3-BAABU, 3-BAABE, paclitaxel, and vinblastine were compared. 3-BAABE treated cells showed nuclear condensation and fragmentation characteristic of apoptosis (Fig. 1A). This observation was also confirmed by comparing 3-BAABU and 3-BAABE using DNA gel electrophoresis (Fig. 2; see below). Cells treated with 3-BAABU did not exhibit characteristics of apoptosis in this time point. The cells possessed intact plasma membranes but nuclear membranes were absent. Most cells contained disorganized chromosomes in a loosely separated and dispersed distribution, suggesting mitotic arrest (Fig. 1B). Cells treated with paclitaxel or vinblastine exhibited initial signs of apoptosis, at less advanced stage than apoptosis induced by 3-BAABE. Paclitaxel showed condensed chromosomes ordered in a concentric belt beneath the plasma membrane (Fig. 1C). Vinblastine demonstrated morphological alterations similar to those of paclitaxel, with even more condensed chromosomes at center of the cells (Fig. 1D).
INHIBITION OF MICROTUBULE ASSEMBLY BY 3-BAABU

Table 2  Phase-specific block of cell cycle at mitosis by 3-BAABU in tumor cells in comparison with its activity in nontumor cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>Block of mitosis</th>
<th>ID&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
<th>ID&lt;sub&gt;90&lt;/sub&gt; (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sp</td>
<td>Human MDS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+</td>
<td>0.017</td>
<td>0.71</td>
</tr>
<tr>
<td>CEM</td>
<td>Human T-cell leukemia</td>
<td>+</td>
<td>0.013</td>
<td>0.02</td>
</tr>
<tr>
<td>PC-3</td>
<td>Human prostate cancer</td>
<td>+</td>
<td>0.05</td>
<td>0.95</td>
</tr>
<tr>
<td>EL4</td>
<td>Murine lymphoma/leukemia</td>
<td>+</td>
<td>0.013</td>
<td>0.02</td>
</tr>
<tr>
<td>B16</td>
<td>Murine melanoma</td>
<td>+</td>
<td>0.07</td>
<td>0.76</td>
</tr>
<tr>
<td>Nontumor cells:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBL</td>
<td>Normal human PBLs</td>
<td>−</td>
<td>&gt;2.0</td>
<td>ND</td>
</tr>
<tr>
<td>MOT</td>
<td>Rat myocardial cells</td>
<td>−</td>
<td>&gt;1.0</td>
<td>2.22</td>
</tr>
<tr>
<td>3T3</td>
<td>Murine fibroblast cells</td>
<td>−</td>
<td>&gt;1.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Shown are the morphological and cytotoxic observation of 3-BAABU in culture. 3-BAABU at various concentrations was incubated with tumor or nontumor cells at 37°C for 48 h. Mitotic arrest is defined as significant increase of mitotic cells (>50%) in comparison with controls.

<sup>b</sup> mDS, myelodysplastic syndrome; ND, not determined.

Table 3  Comparison of the cytotoxicity of 3-BAABU with known antimicrotubule compounds in nonmalignant (MOT) and malignant (CEM) cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>ID&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>ID&lt;sub&gt;50&lt;/sub&gt; (MOT)/ID&lt;sub&gt;50&lt;/sub&gt; (CEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-BAABU</td>
<td>7.00 ± 0.7</td>
<td>104</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>1.94 ± 0.12</td>
<td>81</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>0.93 ± 0.06</td>
<td>62</td>
</tr>
</tbody>
</table>

<sup>a</sup> For determination of ID<sub>50</sub>, see “Materials and Methods.”

Fig. 3. Time kinetics of effects of 3-BAABU in CEM leukemia cells. CEM cells were treated with 3-BAABU (0.05 µg/ml) for 15, 30, 60, 120, or 240 min followed by washing. Viable cells were counted after 48 h of incubation.

The morphological effects of 3-BAABU were also observed in several other tumor cell lines (not illustrated). No visible changes were observed in normal human lymphocytes (2 µg/ml), proliferative rat myocardial cells (MOT; 1 µg/ml), and murine fibroblast cells (3T3; 1 µg/ml). In contrast, mitotic arrest was clearly visible using PC-3 human prostate cancer cells (0.1 µg/ml), murine melanoma cells (0.25 µg/ml), and SP human lymphoid leukemic cells (0.025 µg/ml).

Induction of Apoptosis in Leukemic Cells by 3-BAABU. The time course of the apoptotic effect of 3-BAABU and 3-BAABE on CEM cells was studied comparatively using gel electrophoresis. DNA fragmentation was not seen in 3-BAABU after 4 and 12 h treatment (Fig. 2A, Lanes 2 and 3). Slight DNA degradation appeared after 24 h treatment (Fig. 2A, Lane 4), and a clear multiple-unit ladder pattern of apoptotic DNA was observed at 48 h (Fig. 2A, Lane 5). No mitotic block was found in 3-BAABE treated cells at any time (1, 4, 12, 24, and 48 h after treatment). Instead, a clear apoptotic DNA ladder was detected as early as 12 h after treatment and remained visible at 24 and 48 h (Fig. 2B, Lanes 3–5).

Cancericidal Activity of 3-BAABU. 3-BAABU was highly effective and selective in inducing mitotic arrest and apoptosis in malignant cells. The ID<sub>50</sub> was 0.013 µg/ml for leukemia cells, 0.017 µg/ml for lymphoma, 0.05 µg/ml for prostate cancer cells, and 0.07 µg/ml for melanoma (Table 2). 3-BAABU failed to induce mitotic arrest or apoptosis in normal human lymphocytes, proliferative fibroblast cells (3T3) or myocardial cells (MOT). ID<sub>90</sub> values for leukemia and lymphoma cell lines were 60 to 154 times lower than for nonmalignant cells. Comparison of the cytotoxicity of 3-BAABU with that of known antimicrotubule compounds in nonmalignant cells (MOT; proliferative in culture with doubling time of 24 h approximately) and malignant cells (CEM) showed that the ratio of ID<sub>50</sub> (MOT)/ID<sub>50</sub> (CEM) of 3-BAABU was higher than that of paclitaxel and vinblastine (Table 3).

The effect of the exposure time on cancericidal activity was tested by measuring viability (% of untreated control) immediately after exposure to 3-BAABU for increasing periods of time, and also after a 48 h incubation following the removal of the drug and resuspension of the cells in a fresh, drug-free medium. Cell viability was not immediately affected after exposure at any exposure time up to 4 h.

Fig. 4. Suspending cell cycle at G<sub>1</sub>-M phase in CEM cells treated with 3-BAABU. Cell cycle analysis of CEM cells treated with 3-BAABU (0.05 µg/ml) for 15, 30, 60, 120, or 240 min followed by washing. Viable cells were counted after 48 h of incubation.
INHIBITION OF MICROTUBULE ASSEMBLY BY 3-BAABU

Table 4 Blocking of cell cycle at G2-M by 3-BAABU in CEM leukemic cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Length of Treatment (n)</th>
<th>G0-G1 (%)</th>
<th>S (%)</th>
<th>G2-M (%)</th>
<th>Apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated cells</td>
<td>49</td>
<td>35</td>
<td>14</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Solvent-treated</td>
<td>24</td>
<td>50</td>
<td>34</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>3-BAABU (0.025 µg/ml)</td>
<td>1</td>
<td>48</td>
<td>29</td>
<td>21</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>35</td>
<td>42</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>28</td>
<td>39</td>
<td>29</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>16</td>
<td>35</td>
<td>44</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>4</td>
<td>29</td>
<td>60</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>2</td>
<td>10</td>
<td>70</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1</td>
<td>5</td>
<td>61</td>
<td>33</td>
</tr>
</tbody>
</table>

* CEM cells were incubated with or without 3-BAABU for a predetermined period of time as indicated in the table. Cell cycle analyses were performed as described in "Materials and Methods."

However, an exposure of only 15 min resulted in 30% viability after 48 h incubation (Fig. 3). Increasing exposure time to 60 min reduced viability to 19%. Further increase in the exposure time, up to 240 min, resulted in additional decline, albeit at a very slow rate, down to 7%.

Arrests of CEM Cells at Spindle Assembly Checkpoint by 3-BAABU. CEM cells treated with 3-BAABU were analyzed for their cell cycle. Untreated CEM cells showed classical pattern of proliferating cells proportionally distributed in G0-G1 (50%), S (34%), and G2-M (13%) phases (Fig. 4 and Table 4). 3-BAABU induced a major shift from G0-G1 to G2-M phase. The detectable increase of G2-M phase cells was observed as early as 4 h posttreatment and continuously increased with a reduction of the cells in G0-G1 phase from 50 to 1%. Most cells were accumulated at G2-M phase by 12 h, and the apoptotic cell proportion rose thereafter. These changes were

Fig. 5. Interference with the formation of mitotic spindles in CEM cells by 3-BAABU. The effect of 3-BAABU on formation of microtubules and mitotic spindles in M-phase cells was examined by indirect immunofluorescent staining using anti-β-tubulin monoclonal antibody and FITC-labeled goat antimouse IgG antibody. A, CEM cells were cultured in the absence of 3-BAABU, and mitotic spindles were seen in M-phase cells (one such cell is shown in the middle of the figure); B, CEM cells arrested at mitosis by 3-BAABU (0.025 µg/ml for 24 h at 37°C) show complete absence of mitotic spindles (oil immersion).
consistent with morphological observations and with DNA fragmentation (Fig. 2A).

Disruption of M-phase Mitotic Spindle Formation by 3-BAABU. The distribution of β-tubulin and the formation of mitotic spindles in the M phase of the cells treated with 3-BAABU was studied by immunofluorescent staining. The control consisted of untreated CEM cells, which had about 8–12% cells in M phase and majority of the cells in interphase. Without immunofluorescent staining the background fluorescent was dark. Immunofluorescent staining clearly showed microtubule spindles in untreated mitotic cells (one such cell is shown in the center of Fig. 5A). Upon examination of several thousand mitotic cells arrested by 3-BAABU, none of them showed positive staining for mitotic spindles or astral microtubules (Fig. 5B). Table 5 shows the results of two independent experiments indicating the absence of formation of mitotic spindle in 3-BAABU treated CEM cells.

Effect on Assembly-Disassembly Cycles of Tubulin by 3-BAABU. The kinetics of microtubule assembly and disassembly of control, 3-BAABU, vinblastine, and paclitaxel are shown in Fig. 6. Untreated tubulins exhibited a temperature dependent assembly-disassembly cycle so long as Mg²⁺ and GTP were present. 3-BAABU significantly inhibited the microtubule assembly process but did not affect disassembly. Complete inhibition of microtubular polymerization was caused by 3-BAABU at a concentration as low as 1.0 μg/ml (3.3 μM), whereas microtubular depolymerization was not influenced even when the concentration of 3-BAABU was increased to 20 μg/ml (66 μM). 3-CAABU exhibited inhibitory activity on assembly but not on disassembly, only at the concentrations >25 μg/ml. The analogues 3-BAABE, 4-BAABU, and 3-BPABU resulted in no inhibition on the assembly-disassembly cycle (Table 1). Vinblastine showed a similar mechanism of action as 3-BAABU. In contrast, paclitaxel, which showed no activity on microtubular polymerization, exhibited significant inhibition of the process of microtubule depolymerization.

DISCUSSION

We have synthesized several hundred new compounds in searching for regulatory agents for cell cycle checkpoints, apoptosis, and cell differentiation. Among these, we found a new family of compounds targeting cellular microtubules and therefore regulating cell cycle. The representative compound is 3-BAABU. It causes accumulation of cells in mitosis. Chromosomes in these cells were separated and appear to be randomly distributed in the cytoplasm. Replacing the bromine with chlorine (3-CAABU) or extending the side chain with one more methylene group to form 3-BPABU largely or completely eliminated the mitotic blocking activity. Replacing the urea group with an ethyl ester (3-BAABE) removed the mitotic blocking activity and caused classical apoptosis; moving the bromoacetylamino group to the para- position on the benzene ring (4-BAABU) caused necrotic cell death. These comparisons of the bioactivity among analogues indicate a chemical restriction to the structure of 3-BAABU that is essential for its unique activity of blocking mitosis in tumor cells. The data suggest that the R1 group, the bromoacetylamino chain, is critical for the cytotoxicity, and the R2 group, a urea, as well as its meta-

relationship to bromoacetylamino (R1) plays a significant role in regulating the action. The lower activity of the analogues, with respect to 3-BAABU, could result from their three-dimensional configurations, which presumably have poor affinity to cellular components. Morphological evidence of apoptosis by 3-BAABE (Fig. 1A) was

![Fig. 6. The effect of 3-BAABU on heat-dependent polymerization-depolymerization cycle of microtubules in a cell-free system. A, free tubulins in reaction buffer were incubated with GTP and Mg²⁺ at 37°C for the assembly process in the absence or presence of 3-BAABU (3.3 μM), paclitaxel (23 μM), or vinblastine (22 μM); B, for the disassembly process, assembled microtubules were incubated in ice in the absence or presence of 3-BAABU or paclitaxel or vinblastine at concentrations mentioned above. The level of the microtubule assembly and disassembly was measured every 5 min by absorbance at 350 nm. Error bars are not shown to preserve clarity. SD values (n = 3) were in the 0.008–0.015 range.](image-url)
supported by DNA gel analyses. Because 3-BAABE failed to induce mitotic arrest at any time points examined and failed to inhibit microtubule assembly-disassembly process at concentrations between 0.1 and 100 μM, this compound probably triggers apoptotic cascade through some unknown mechanisms rather than by mitotic arrest. DNA degradation induced by 3-BAABE commenced within 12 h after treatment, in contrast to that of 3-BAABU, suggesting a shorter pathway of 3-BAABE.

The time course experiments in CEM cells showed a 2-step process of 3-BAABU activity. In the first 24 h, 3-BAABU suspended the cell cycle in early or middle M phase. In the second step, between 24 and 48 h, cells that were accumulating in M phase entered into apoptotic pathway, as shown by DNA fragmentation in gel electrophoresis.

Furthermore, the uptake of 3-BAABU by cells was rapid. Cell viability was not effected immediately after exposure regardless of exposure time (up to 4 h), indicating that prolonged incubation was needed for the cytotoxicity, as described above. Indeed, an exposure of only 15 min resulted in 30% viability after a 48-h incubation (Fig. 3), in accordance with the time needed for the cells to enter the M phase, where the antimicrotubule activity of the drug commenced. Most of the cytotoxicity was commenced after the first 60 min of exposure (19%), although additional significant decrease in viability, albeit at a slow rate, was observed down to 7% viability at 240 min of exposure (Fig. 3).

The data presented in Tables 2 and 3 show a significant difference in cytotoxicity of 3-BAABU for malignant and proliferative nonmalignant cells. ID

| 100 |  values of 3-BAABU in the proliferative nonmalignant cells were about 60–150 times higher than those for leukemia or lymphoma cells, indicating a highly selective toxicity of 3-BAABU. Although this phenomenon was also observed in other antimicrotubule agents, the selectivity of 3-BAABU was better than that of paclitaxel and vinblastine. Because 3-BAABU inhibits repolymerization of tubulin, the preferential action against tumor cells is important. The relationships between microtubules and products of oncogenes/tumor suppressor genes have been reported (14–18). The perturbation of microtubules may signal events that disrupt proliferative predominance in tumor cells. It has been reported recently that antimicrotubule agents such as Taxol and Vinca alkaloids induce Raf-1/bcl-2 phosphorylation following disruption of microtubular architecture (19), and the phosphorylation of bcl-2 inhibits its binding to bax, selectively leading the bcl-2 positive tumor cells to apoptosis (20). An alternative explanation assumes structural differences between normal and tumor cell microtubules or microtubule-associated proteins, such as MAP2 or tau (21, 22).

Cell cycle analyses showed a major accumulation of G2-M (4n) cells induced by 3-BAABU, concurrent with a significant reduction of cells in G1,S, G2 (2n) and S phase (2n–4n). Considering the cell cycle analyses together with morphological findings, it appeared that the 4n peak at 12 h contained a mixture of G2 and M cells, all of which then progressed into and arrested at prometaphase/metaphase/anaphase between 12–24 h. After microscopic examination of over 50,000 cells, no instance of telophase was seen. Because more than 90% of the cells were in mitosis, this indicates a failure of downstream processes at prometaphase/metaphase/anaphase, which may correspond to up-regulation of the M-phase controller, e.g., the spindle assembly checkpoint (23–25). One of the control mechanisms of the M-phase checkpoint is the alignment of chromosomes on mitotic spindles for cell division (26, 27). The spindle assembly checkpoint inhibits the onset of anaphase in cells that lack mitotic spindles (26, 27). The morphological features of 3-BAABU in leukemic cells showed a disorientation of M-phase chromosomes in the cytoplasm. It was therefore speculated that if the mitotic arrest was a result of interference with mitotic spindle formation, 3-BAABU is most likely an antimicrotubule agent. This was confirmed by two experiments discussed below.

Two distinct antimicrotubule mechanisms have been identified among antimicrotubular agents: (a) inhibiting the polymerization of tubulin, which destabilizes microtubules, e.g., Vinca alkaloids and colchicine (1, 28); and (b) promoting the assembly of tubulin polymer and forming abnormal and excessively stable microtubule structures (1, 29), e.g., Taxol. Immunofluorescence assay with anti-β-tubulin monoclonal antibodies demonstrated a complete absence of mitotic spindles or mitotic astrals in the M-phase cells arrested by 3-BAABU, in contrast to the untreated cells which showed mitotic spindles in about 7–8% cells at various stages of mitosis. Because the cytoplasm showed homogenous staining of β-tubulin, it was assumed that 3-BAABU interfered with the assembly of microtubules from tubulins, thus preventing the formation of mitotic spindles. The experiments of inhibition of repolymerization of tubulin in the cell-free system demonstrated that 3-BAABU abrogated the process of assembly of microtubule from tubulin but did not interfere with the disassembly of microtubules. The results suggest that the mechanism of action of 3-BAABU is similar to that of vinblastine and is consistent with immunofluorescent staining, which showed an absence of mitotic spindles. It is likely that 3-BAABU blocks the assembly process of the heat-dependent polymerization-depolymerization cycle by direct binding to tubulin at the sites necessary for repolymerization of tubulin. We have obtained a significant inhibition of microtubule assembly at concentrations of 3-BAABU as low as 0.35 μM, indicating that its affinity to tubulin is similar to that of vinblastine, podophyllotoxin, and colchicine (30, 31).

The facts that paclitaxel inhibits the depolymerization of microtubule and morphologically exhibits "aster" (G2-M phase) and "bundles" (throughout the cell cycle) structures of microtubules (32–34) clearly distinguish 3-BAABU from paclitaxel. Whether the mechanism of action of 3-BAABU is similar to or different from vinblastine is of mechanistic importance. In immunofluorescent staining, vinblastine induced abnormal spindles or punctuate aggregates of tubulin in target cells (35). Such microtubular structures were not observed in 3-BAABU treated cells. Additionally, morphological observation revealed different characteristics of changes in chromosomes between 3-BAABU and vinblastine. There is evidence that paclitaxel and the Vinca alkaloids are substrates for PGP (36, 37). Using Daudi/Pgp+ and Daudi/Pgp− cell lines, we found that 3-BAABU was apparently not a substrate for PGP. Therefore, although the antimicrotubule effect of 3-BAABU and of vinblastine is due to inhibition of repolymerization, there must be different mechanisms of action of these compounds.

Because 3-BAABU interferes with the process of microtubule assembly selectively in malignant cells, prevents formation of mitotic spindles, suspends cell cycle at M phase, and eventually leads tumor cells to apoptosis, there is justification for the development of this new antimicrotubule compound as a potential anticancer agent.


Inhibition of Microtubule Assembly in Tumor Cells by 3-Bromoacetylamino Benzoylurea, a New Cancericidal Compound

Jian-Dong Jiang, Yue Wang, John Roboz, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/58/10/2126

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.